Panfungal PCR assays using fresh frozen paraffin embedded tissue specimens for fungal species identification and the detection of azole-resistance mutations in the A. fumigatus cyp51A gene at a South Korean hospital

In Young Jung
Yonsei University Wonju College of Medicine
Youn-Jung Lee
Yonsei University College of Medicine
Hyo Sup Shim
Yonsei University College of Medicine
Woon Ji Lee
Yonsei University College of Medicine
Jun Hyoung Kim
Yonsei University College of Medicine
Hye Seong
Yonsei University College of Medicine
Jung Ho Kim
Yonsei University College of Medicine
Jin Young Ahn
Yonsei University College of Medicine
Su Jin Jeong
Yonsei University College of Medicine
Nam Su Ku
Yonsei University College of Medicine
Yoon Soo Park
Yonsei University College of Medicine
Joon Sup Yeom
Yonsei University College of Medicine
Young Keun Kim
Yonsei University Wonju College of Medicine
Hyo Youl Kim
Yonsei University Wonju College of Medicine
Jun Yong Choi (seran@yuhs.ac)
Yonsei University College of Medicine https://orcid.org/0000-0002-2775-3315

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Abstract

Background: With rising concerns about changing fungal epidemiology and azole resistance in Aspergillus species, identifying fungal species and susceptibility patterns of mucorales and aspergillosis are crucial in the management of these diseases. The objectives of this study were to evaluate performance of panfungal PCR assays on formalin-fixed paraffin embedded (FFPE) samples for fungal species identification, and the detection of azole-resistance mutations in the Aspergillus fumigatus (A.fumigatus) cyp51A gene at a South Korean hospital.

Methods: A total of 75 FFPE specimens with a histopathological diagnosis of aspergillosis or mucormycosis were identified during the 10-year study period (2006-2015). After deparaffinization and DNA extraction, panfungal PCR assays were conducted on FFPE samples for fungal species identification. The identified fungal species were compared with histopathological diagnosis. On samples identified as A.fumigatus , sequencings to identify frequent mutations in the cyp51A gene (tandem repeat 46 [TR46], L98H, and M220 alterations) that confer azole resistance were performed.

Results: Specific fungal DNA was identified in 31 (41.3%) FFPE samples, and of these, 16 samples of specific fungal DNA were in accord with histopathological diagnosis of aspergillosis or mucormycosis. 15 samples had discordant histopathology and PCR results. No azole-mediating cyp51A gene mutation was revealed among nine cases of A. fumigatus . Moreover, no cyp51A mutations were identified among three cases with history of prior azole use.

Conclusion: The pan-fungal PCR assay with FFPE sample may provide additional information on fungal species identification. No azole-resistance mediating mutations in the A. fumigatus cyp51A gene were identified among FFPE samples during study period.

Background

Mucormycosis (formerly known as zygomycosis) and aspergillosis are invasive fungal diseases that usually present as rhino-orbital-cerebral or pulmonary infections. [1, 2] *Aspergillus* species are usually susceptible to voriconazole, and isavuconzole has also become a first-line targeted therapy[3], whereas voriconazole has no activity agents mucorales. [3] Moreover, concerns about changing epidemiology and azole resistance are rising.

Higher rates of mortality have been demonstrated for patients treated with voriconazole in voriconazole-resistant invasive aspergillosis (IA) than for voriconazole-susceptible IA [4, 5]. Rapid detection of fungal species and of azole-resistance in *Aspergillosis fumigatus (A. fumigatus)* may benefit outcomes by guiding appropriate antifungal therapy. [4]

Azoles are inhibitors of 14α-sterol demethylases, which are responsible for catalyzing a critical step in the biosynthesis of ergosterol a component of fungal membrane. [5] Mutation in the *cyp51A* gene, which is responsible for encoding 14α-sterol demethylase enzymes, is the most common azole-resistance mechanism in *Aspergillus* species. [6] Moreover, isolates harboring tandem repeats (TRs) in the promoter region of the *cyp51A* gene and point mutations leading to amino acid changes are also known to cause azole-resistance. [6] Furthermore, the incidence of azole-resistant *Aspergillus* species has increased over recent years due to previous exposure and environment-associated resistance. [5, 7]

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Molecular methods can now be used to rapidly identify fungal species in Formalin-Fixed and Paraffin-Embedded (FFPE) tissue specimens [8], and Polymerase Chain Reaction (PCR) based methods have been devised to detect azole-resistance in FFPE and bronchial alveolar lavage specimens. [9, 10] The objectives of this study were to evaluate performance of panfungal PCR assays on FFPE samples for fungal species identification, and the detection of azole-resistance mutations in the *Aspergillus fumigatus* (*A.fumigatus*) cyp51A gene at a South Korean hospital..

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Results

During the 10-year study period, 75 patients received a histopathological diagnosis of mucormycosis or aspergillosis, and PCR amplification and identification was positive for 31 (41.3%) of the 75 FFPE samples.

Sixteen FFPE samples had corresponding histopathology and PCR sequencing results. Fourteen cases of *Aspergillus* species were identified; *A. fumigatus* (n = 9), *A. flavus* (n = 2), *A. oryzae* (n = 2), and *A. tamarii* (n = 1). Two cases with a histopathological diagnosis of mucormycosis were identified as *Rhizopus oryzae* by sequence analysis. The nine cases identified as *A. fumigatus* species were further analyzed for azole-resistance mutations in the *A. fumigatus cyp51A* gene.

The demographic and clinical data of the 16 patients identified as aspergillosis or mucormycosis by panfungal PCR are presented in Table 1. Patient 1, a 77-year-old male, had a history of chronic obstructive pulmonary disease (COPD) and was receiving steroids when he developed a brain abscess. Empirical antibacterial agent, but no antifungal agent, was administered. Aspergillosis was confirmed after death by pathologic diagnosis, and PCR sequencing confirmed *A. fumigatus.* Patient 2 was an 81-year-old male patient who developed fungal pneumonia after surgery for renal cell carcinoma. Pathologic diagnosis conducted on transbronchial lung biopsy tissue revealed aspergillosis. The patient was treated with voriconazole but succumbed despite appropriate treatment. PCR sequencing identified *A. fumigatus.* Patient 4 was a 75-year-old male patient who had undergone liver transplantation due to hepatocellular carcinoma and was receiving immunosuppressive therapy and oral itraconazole for fungal prophylaxis prior to developing acute maxillary sinusitis. Patient 5 was a 62-year-old female and had undergone liver transplantation due to liver cirrhosis. A mass lesion developed in her chest wall area 1 year after transplantation. Excisional biopsy revealed narrow branching fungal hyphae consistent with aspergillosis, and PCR sequencing demonstrated the presence of *A. fumigatus*. The patient was receiving fluconazole for fungal prophylaxis. Treatment, which involved excision and systemic voriconazole, was successful in this case. Patient 9 was a 54-year-old male and had undergone a lung transplant due to idiopathic pulmonary fibrosis. He had a history of itraconazole use for fungal prophylaxis. Patient 15 was a 62-year-old male that had undergone kidney transplantation due to diabetic nephropathy. Histopathologic diagnosis after stomach biopsy revealed mucormycosis. Despite treatment with liposomal amphotericin B, the patient expired. PCR sequencing results identified *Rhizopus oryzae*. Patient 16 was a 69-year-old male with diabetes mellitus on insulin therapy when he developed maxillary sinusitis. Mucormycosis was confirmed by histopathology and was consistent with PCR results, which demonstrated *Rhizopus oryzae*.

The results of the *cyp51A* alterations of the nine samples confirmed as *A. fumigatus* are summarized in table 1. Seven samples were positive by the L98H PCR assay alone, but no mutations were detected by sequence analysis. Seven samples were positive by the M220 PCR assay alone, but also revealed no mutations by sequence analysis. Six samples were positive by the TR 46 PCR assay alone, but similarly, no mutations were revealed by sequence analysis.

Discordant PCR and histopathology results were obtained for 15 samples. Two specimens histopathologically diagnosed as Aspergillosis were identified as mucorales; *Lichtheimia ramose* and *Rhizopus oryzae.* Five specimens with a histopathological diagnosis of aspergillosis were identified by PCR as *Epicoccum nigrum*, *Bipolaris zeicola*, *Fusarium solani*, *Nakataea oryzae* and

Cladosporium cladosporioides. Eight samples were identified by PCR sequencing as uncultured fungus clones. One brain sample diagnosed as mucormycosis by histopathology was identified by PCR as an uncultured fungus clone. *Lichtheimia ramose* was identified by PCR in buttock tissue. *Rhizopus oryzae, Bipolaris zeicola*, *Fusarium solani*, *Nakataea oryzae* were all identified in sinus samples with uncultured fungus clones, and *Epicoccum nigrum* and *Cladosporium cladosporioides* were identified in lung samples.

Discusison

The identification of fungal DNA in tissue samples by PCR improves diagnostic accuracies for fungal infections [3], and pan-fungal PCR conducted on FFPE tissues provides an alternative to culture dependent identification methods. [8] Mucorales has been identified by PCR in paraffin-embedded tissue samples of patients with a fungal infection [11, 12], and it has been shown fungal organisms can be identified by amplifying fungal ITS 1 and 2 using pan-fungal primers. [12] The results of the present study concur that PCR amplification of the ITS 1 and 2 regions accurately diagnoses fungal species in FFPE specimens.

In all 31 FFPE samples that produced amplifiable DNA results, fungi were identified to the genus or species level. In 2 cases with a histopathologic diagnosis of aspergillosis, mucorale specific DNA was identified by sequencing PCR products. Although this may have been due to tissue specimen contamination, the risk of misdiagnosis by histopathology cannot be excluded. Similar cases have been described in cases confirmed by culture. [13]

Two samples with a histopathologic diagnosis of aspergillosis were identified as mucorales; *Lichtheimia ramose and Rhizopus oryzae.* The *Lichtheimia* species (formerly known as *Absidia)* are currently regarded as emerging pathogens among Mucoralean fungi.[14] In the present study, the male patient identified with *Lichtheimia ramose* infection had a history of hepatocellular carcinoma and had undergone liver transplantation prior to infection. In addition, he was under immunosuppressive medication. Biopsy from a buttock revealed mucormycosis by PCR product sequencing. Although it is generally known to have low virulence, cases of mucormycosis due to *Lichtheimia ramose* in immunocompromised hosts have been reported. [14, 15] Chaumont et al. reported a case of cutaneous mucormycosis requiring aggressive surgical debridement. [16]

The second case, initially diagnosed by histopathology as aspergillosis, was found to be due to *Rhizopus oryzae* by PCR. This patient had a history of aplastic anemia before fungal infection and displayed rapid clinical deterioration resulting in death. *Rhizopus oryzae* is the most common cause of zygomycosis, and is a life-threatening infection that usually occurs in patients with diabetic ketoacidosis. [17]

Four samples histopathologically diagnosed as Aspergillosis produced ambiguous results. In addition to PCR results corresponding as *Aspergillus* species, 3 samples a concomitant uncultured fungus was identified and in the other sample *Nakataea oryzae* strain was identified. Because fungal ribosomal genes have many similarities, identification at the species level can only be performed by sequencing PCR products. [18] However, it has been shown that even targets of base pairs of less than 300 bp within 18S rDNA may not be sufficient to differentiate genera. [19] Furthermore, *A. fumigatus* cannot be identified at the species level by PCR targeting 18S rDNA, because target sequences show high analogy to several Ascomycota. [19] We believe this lack of specificity may have explained the uncultured fungus clones identified in the present study.

Since aspergillosis and mucormycosis responds to different antifungal agents, delayed diagnosis or treatment might lead to devastating results. [20] Mucormycosis is an aggressive and invasive disease, and the early surgical debridement of involved tissues and initiation of proper antifungal agents are crucial. [21] Our study results demonstrate that PCR can be used to differentiate and identify fungal species, and thus, provide guidance regarding appropriate antifungal treatment strategies.

Mutations in the *cyp51A* gene have been documented in clinical isolates of patients with a long history of exposure to azoles. [22, 23] The most frequent resistance allele is TR34 in combination with L98H substitution (TR34/L98H). [5] Azole-resistant mutations harboring variously sized tandem repeats in the promoter region of the *cyp51A* gene and point mutations leading to amino acid changes in the *cyp51A* gene have also been documented in azole-naive patients. [24, 25] TR34/L98H and TR46/Y121F/T289A are commonly associated with azole-resistance linked to environmental use of azoles in agriculture, and often found in azole-naïve patients. [26, 27] In the present study, azole resistance was not detected in three cases (patients 4, 5, and 9) with a history of prior azole use. Thus, because sample numbers were small, we suggest larger scale studies be performed to investigate azole resistance in patients with a history of azole exposure.

Several limitations of the present study warrant mention. First, the amount of fungal DNA available is crucial when investigating clinical samples, and DNA degradation and the effects of formaldehyde may have reduced DNA amounts in samples. Second, culture results or azole susceptibility profiles were not considered. Third, as the TR46 and M220 mutations have never been reported in South Korea, a positive control for isolates harboring these mutations could not be acquired. And finally, there are many mutations within *cyp*51A that can confer elevated MICs/resistance to the triazoles, not just the ones stated in this paper, as well there areas unknown methods of elevated MICs to the triazoles not linked to *cyp*51A mutations that would not be detected by this assay.

Conclusion

The pan-fungal PCR assay with FFPE sample may provide additional information on fungal species identification. No azole-resistance mediating mutations in the A. fumigatus cyp51A gene were identified among FFPE samples during study period.

Methods

Clinical samples and DNA extraction

Histopathology reports consistent with aspergillosis or mucormycosis issued between January 2006 and January 2016, and relevant FFPE blocks were retrieved from the Department of Pathology at a tertiary referral hospital in South Korea. Ethics approval was obtained from the hospital's institutional review board (Yonsei University Health System, Severance Hospital, Institutional Review Board, IRB trial number: 4-2016-0262). Pan-fungal PCR assays were performed on these FFPE blocks to determine the presence of *Aspergillus* species and mucorales. Samples testing positive for *A. fumigatus* by DNA sequencing were subjected to L98H, M220, and TR46 PCR assays and consecutive DNA sequence analysis to determine the presence of azole-resistance mutations in the *A. fumigatus cyp51A* gene.

FFPE tissues were deparaffinized with mineral oil, and DNA was extracted with proteinase K using the ReliaPrep™ FFPE gDNA Miniprep System (Promega, Madison, WI, USA), according to the manufacturer's instructions. Agarose gel electrophoresis and PCR methods were used to assess DNA degradation.

Primers for PCR assays of Aspergillus species and Mucormycosis identification

Pan-fungal PCR was performed to amplify internal transcribed spacer (ITS) regions. The primers ITS5 (forward; 5'GGAAGTAAAAGTCGTAACG-3') and ITS4 (reverse; 5'- TCCTCCGCTTATTGATATGC - 3') were used to amplify ITS 1 to ITS 2 regions (ITS 1-2), and the primers ITS3 (forward; 5'- GCATCGATGAAGAACGCAGC -3') and ITS4 (reverse; 5'- TCCTCCGCTTATTGATATGC - 3') were used to amplify the ITS 2 region. [12] The ITS 1-2 and ITS 2 PCR products so obtained were of 640 and 350 base pairs (bp), respectively. (supplementary table 1)

Primers for PCR assays of cyp51A gene mutations

To amplify L98H, M220, and TR46 mutation in the *cyp51A* gene, we used a previously described nested, one-step PCR assay. [28] Three different primer sets were used to amplify these three mutations. To amplify L98H, we used 5'- AAAAAACCACAGTCTACCTGG - 3' (forward), and 5'- GGAATTGGGACAATCATACAC - 3' (reverse) to generate a 143 bp PCR fragment. [29] For M220, we used 5'- GCCAGGAAGTTCGTTCCAA - 3' (forward) and 5'- CTGATTGATGATGTCAACGTA - 3' (reverse) to generate a 173 bp PCR fragment. [29] Nested PCR assay was performed to amplify TR46 in the promoter region of *cyp51A*; one primer pair was used to amplify a long DNA fragment and a second primer pair was used to amplify an inner shorter fragment in a second PCR step. For the first step, the PCR primer pairs were 5' - AAGCACTCTGAATAATTTACA - 3' (forward) and 5' - ACCAATATAGGTTCATAGGT - 3' (reverse) to obtain a 240 bp DNA fragment, and in the second step, 5' - GAGTGAATAATCGCAGCACC - 3' (forward) and 5' - CTGGAACTACACCTTAGTAATT - 3' (reverse) were used to generate a 103 bp DNA fragment. [28] (supplementary table 2)

PCR assays and controls

To amplify ITS regions, PCR was performed in total volumes of 50 µl, consisting of 1X reaction buffer, 0.1 µM dNTPmix, 1.25 U of Taq DNA Polymerase (RBC Bioscience, Xindian City, Taiwan), 20 pmol of each primer, and 200 ng of DNA (1 µL) per sample. PCR was performed using the following protocol; 95°C for 3 minutes, 35 amplification cycles of 94°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 7 minutes.

To detect L98H and M220 alterations, PCR was conducted in a total volume of 50 µl containing 2 µl template DNA (100 ng human DNA + unknown amount of A. fumigatus DNA), 1X reaction buffer, 0.1 µM dNTPmix, 1.25 U of Taq DNA Polymerase (RBC Bioscience, Xindian City, Taiwan), and 20 pmol of each primer. The PCR amplification protocol was as follows; 5 min of initial denaturation at 94°C, 39 amplification cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min.

To detect TR46 alteration**s,** PCR was conducted in a total volume of 50 µl as described for L98H and M220 above. The PCR amplification protocol was as follows; 5 min denaturation at 94°C, 22 amplification cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min. For the second PCR step, we used a total volume of 50 µl and 3 µl of the first-step PCR mixture as template. Other components were as described for L98H and M220. The second step PCR amplification protocol was as follows; 5 min initial denaturation at 94°C, 34 amplification cycles of 94°C for 45s, 56°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min.

To exclude cross-reactivity of the primers with human genomic DNA, samples containing a mixture of 100 ng of human DNA and 50 pg of *A. fumigatus* wild-type DNA were used as a negative control. An azole-resistant *A. fumigatus* strain (GenBank accession no. AF338659) harboring the TR34/L98H/S297T/ F495L mutation in the *cyp51A* gene was used as a positive control for detection of the L98H mutation.[30]

Sequence analysis

To identify *Aspergillus* species and mucorales, PCR products were purified using the MiniElute PCR purification kit (Qiagen, Hilden, Germany). A minimum of 50 ng DNA was sequenced using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an Applied Biosystems 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were edited and aligned using Sequence Scanner Software 2 ver. 2.0 (Applied Biosystems, Foster City, CA), and product sequences were compared with reference sequences using the NCBI alignment service AlignSequenNucleotideBlast (http://www.ncbi.nlm.nih.gov/). The GenBank accession number for the *A. fumigatus* sequences determined in this study is CM000169.1.

To detect potential mutations in the PCR products subjected to DNA sequence analysis, sequences were compared with the *A. fumigatus cyp51A* wild-type sequence using the NCBI alignment service AlignSequenceNucleotideBlast (http://www.ncbi.nlm.nih.gov/).

Declarations

Ethics approval and consent to participate

Ethics approval was obtained from the hospital's institutional review board (Yonsei University Health System, Severance Hospital, Institutional Review Board, IRB trial number: 4-2016-0262).

Consent for publication

No applicable

Availability of data and materials

The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

I.Y.J. and J.Y.C. conceived the ideas; H.S.S. collected the data; Y.J.L. conducted the experimental design, W.J.L, J.H.K., H.S., J.H.K., J.Y.A., S.J.J., N.S.K., Y.S.P, J.S.Y., Y.K.K., and H.Y.K. analysed the data; and I.Y.J. and J.Y.C. led the writing. All authors have read and approved the manuscript

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Tables

Table 1. Demographics of the 16 cases identified as aspergillosis or mucormycosis and *Cyp51A* alterations of the nine cases sequenced as *Aspergillus fumigatus*

Case	Sex /age	Underlying condition	Type of tissue	Histopathologic identification	Molecular identification	Culture	Serum GM	Prior azole use	Mortality	*cyp51A* alterations		
										L98H	M220	TR46
1	M/77	COPD	Brain	Aspergillosis	*A.fumigatus*	NG	Neg	No	Yes	Neg	Neg	NT
2	M/81	Renal cell carcinoma	Lung	Aspergillosis	*A. fumigatus*	NG	Neg	No	Yes	Neg	Neg	Neg
3	M/62	Chronic sinusitis	Maxillary sinus	Aspergillosis	*A. fumigatus*	NT	NT	No	No	Neg	NT	NT
4	M/75	Liver transplantation †	Maxillary sinus	Aspergillosis	*A. fumigatus*	NT	Neg	Yes	No	Neg	Neg	NT
5	F/62	Liver transplantation †	Soft tissue/bone ¶	Aspergillosis	*A. fumigatus*	NG	Pos	Yes	No	Neg	Neg	Neg
6	F/74	Chronic sinusitis	Maxillary sinus	Aspergillosis	*A. fumigatus*	NT	NT	No	No	NT	NT	Neg
7	F/78	ESRD	Maxillary sinus	Aspergillosis	*A. fumigatus*	NT	NT	No	No	NT	Neg	Neg
8	F/58	Chronic sinusitis	Maxillary sinus	Aspergillosis	*A. fumigatus*	NT	NT	No	No	Neg	Neg	Neg
9	M/54	Lung transplantation ‡	Lung	Aspergillosis	*A. fumigatus*	NG	Pos	Yes	No	Neg	Neg	Neg
10	M/73	Diabetes mellitus	Maxillary sinus	Aspergillosis	*A. flavus*	NT	NT	No	No			
11	M/71	Cervix cancer, CKD	Lung	Aspergillosis	*A. tamarii*	Fungus	Pos	Yes	Yes			
12	M/52	Chronic sinusitis	Maxillary sinus	Aspergillosis	*A. oryze*	NT	NT	No	No			
13	F/62	Chronic sinusitis	Maxillary sinus	Aspergillosis	*A. oryze*	NT	NT	No	No			
14	M/65	ESRD	Maxillary sinus	Aspergillosis	*A. flavus*	NT	NT	No	No			
15	M/62	Kidney transplantation §	Stomach	Mucormycosis	*Rhizopus oryzae*	Fungus		No	Yes			
16	M/69	Diabetes mellitus	Maxillary sinus	Mucormycosis	*Rhizopus oryzae*	NT		Yes	Yes			

GM; serum galactomannan, M, male; F, female; COPD, chronic obstructive pulmonary disease; ESRD, end stage renal disease; CKD, chronic kidney disease; *A.fumigatus, Aspergillus fumigatus*; *A. flavus, Aspergillus flavus*; *A. tamarii, Aspergillus tamarii*; *A. oryze, Aspergillus oryze*; NG, no growth; NT, not tested (insufficient samples remaining); Neg, negative; Pos, positive

† Liver transplantation due to hepatocellular carcinoma

‡ Lung transplantation due to idiopathic pulmonary fibrosis

- Kidney transplantation due to diabetic nephropathy

¶ Biopsies of chest wall soft tissue and left rib

Supplementary table 1. The composition of primer sets and PCR conditions for *Aspergillus* species or mucorales identification

Region	Primer name	Orientation	Nucleotide sequence (5'–3')	Fragment Length (bp)	PCR conditions
ITS 1 to 2 region (ITS 1-2)	ITS 5	Forward	GGAAGTAAAAGTCGTAACG	640	Total vol, 50 µl; 1 µl template DNA, 1X reaction buffer, 0.1 µM dNTPmix, 1.25 U of Taq DNA Polymerase (RBC Bioscience, Xindian City, Taiwan), 20 pmol of each primer, 200 ng of DNA (1 µL); 3 min of initial ampification at 95°C, 35 cycles of 94°C for 30 s, 50°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 7 min
	ITS 4	Reverse	TCCTCCGCTTATTGATATGC		
ITS 2 region (ITS 2)	ITS 3	Forward	GCATCGATGAAGAACGCAGC	350	ITS 2 region PCR conditions are on par with the ITS 1-2 conditions
	ITS 4	Reverse	TCCTCCGCTTATTGATATGC		

PCR, polymerase chain reaction; ITS, internal transcribed spacer regions, bp; base pair

Supplementary table 2. The composition of *A.fumigatus cyp51A*-specific primer sets used and PCR conditions for detecting *cyp51A* mutations

Primer	Orientation	Nucleotide sequence (5'–3')	Fragment Length (bp)	PCR conditions
L98H mutation	Forward	AAAAAACCACAGTCTACCTGG	143	Total vol, 50 µl; 2 µl template DNA (approx. 100 ng human DNA + unknown amount of *A.fumigatus* DNA), 1X reaction buffer, 0.1 µM dNTPmix, 1.25 U of Taq DNA Polymerase (RBC Bioscience, Xindian City, Taiwan), 20 pmol of each primer; 5 min of initial denaturation at 94°C, 39 cycles of 94°C for 45s, 52°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min
	Reverse	GGAATTGGGACAATCATACAC		
M220 mutation	Forward	GCCAGGAAGTTCGTTCCAA	173	M220 PCR conditions are on par with the L98H conditions
	Reverse	CTGATTGATGATGTCAACGTA		
TR46				
Step 1	Forward	AAGCACTCTGAATAATTTACA	240	First step, total vol, 50 µl ; components of PCR mixture are on par with those of the L98H PCR mixture; 5 min initial denaturation at 94°C, 22 cycles of 94°C for 45 s, 52°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min
	Reverse	ACCAATATAGGTTCATAGGT		
Step 2	Forward	GAGTGAATAATCGCAGCACC	103	Second step, total vol, 50 µl; template of 3µl of the first-step PCR mixture; other components are on par with those of the L98H PCR mixture; 5 min initial denaturation at 94°C, 34 cycles of 94°C for 45 s, 56°C for 1min, and 72°C for 1 min, and final extension at 72°C for 5 min
	Reverse	CTGGAACTACACCTTAGTAATT		

A.fumigatus, Aspergillus fumigatus; bp; base pair; TR, tandem repeat